Apoptosis (2008) 13:1450–1464
DOI 10.1007/s10495-008-0271-0

ORIGINAL PAPER

Withaferin A induces apoptosis by activating p38 mitogen-activated protein kinase signaling cascade in leukemic cells of lymphoid and myeloid origin through mitochondrial death cascade

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Published online: 6 November 2008

Abstract Withaferin A (WA) is present abundantly in *Withania somnifera*, a well-known Indian medicinal plant. Here we demonstrate how WA exhibits a strong growth-inhibitory effect on several human leukemic cell lines and on primary cells from patients with lymphoblastic and myeloid leukemia in a dose-dependent manner, showing no toxicity on normal human lymphocytes and primitive hematopoietic progenitor cells. WA-mediated decrease in cell viability was observed through apoptosis as demonstrated by externalization of phosphatidylserine, a time-dependent increase in Bax/Bcl-2 ratio; loss of mitochondrial transmembrane potential, cytochrome *c* release, caspases 9 and 3 activation; and accumulation of cells in sub-G0 region based on DNA fragmentation. A search for the downstream pathway further reveals that WA-induced apoptosis was mediated by an increase in phosphorylated p38MAPK expression, which further activated downstream signaling by phosphorylating ATF-2 and HSP27 in leukemic cells. The RNA interference of p38MAPK protected these cells from WA-induced apoptosis. The RNAi knockdown of p38MAPK inhibited active phosphorylation of p38MAPK, Bax expression, activation of caspase 3 and increase in Annexin V positivity. Altogether, these findings suggest that p38MAPK in leukemic cells promotes WA-induced apoptosis. WA caused increased levels of Bax in response to MAPK signaling, which resulted in the initiation of mitochondrial death cascade, and therefore it holds promise as a new, alternative, inexpensive chemotherapeutic agent for the treatment of patients with leukemia of both lymphoid and myeloid origin.

Keywords Acute lymphoblastic leukemia · Myeloid leukemia · Withaferin A · Apoptosis · Activation of p38 MAPK · Mitochondrial death cascade · Transcription factors

Abbreviations

ALL	Acute lymphoblastic leukemia
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
dUTP	2′-Deoxyuridine 5′-triphosphate
TUNEL	Terminal deoxyneuleotidyltransferase enzyme-mediated dUTP end labeling
FITC	Fluorescein isothiocyanate
JC-1	5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
$\Delta\psi_m$	Mitochondrial trans-membrane potential

Electronic supplementary material The online version of this article (doi:10.1007/s10495-008-0271-0) contains supplementary material, which is available to authorized users.

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MS	Mass spectroscopy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
2D NMR	Two-dimensional nuclear magnetic resonance
^{1}HNMR	^{1}H (proton) Nuclear magnetic resonance
PBMC	Peripheral blood mononuclear cells
PI	Propidium iodide
WA	Withaferrin A
Z-VAD-fmk	Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl keton

Introduction

Chemotherapy for leukemia is generally dependent on combinational treatment, and is still far from producing a satisfactory result especially in underdeveloped countries due to its high cost and prolonged treatment protocol [1]. Clinically, although the rate of complete remission is high among leukemia patients using current chemotherapy protocols in the Western countries, disease-free survival is poor in underdeveloped countries. An alarming increase in the incidence of drug resistance coupled with adverse effects and high treatment costs sets out the need to explore alternative therapeutic agents. Thus, the development of an alternative therapy directly targeting the leukemia cells has become a priority.

Medicinal plants described in Ayurveda and traditional Chinese literatures are the key knowledge resources that inspired molecular research for plant-based drug development world over. These natural compounds have shown promising outcomes in cancer therapy for the past 30 years. These products provide many lead structures, which are subsequently used in the design of novel compounds with enhanced biological properties. Therefore, the examination of therapeutic potential of characteristic phytochemicals forms an important facet of anti-cancer drug discovery because medicinal plants have a very long history of safe consumption, and bioactive compounds obtained from them are normally non-toxic or less toxic to humans [2].

Ashwagandha (*Withania somnifera* Dunal, Solanaceae) is one of the most reputed medicinal herbs that forms an essential constituent of over 100 traditional medicine formulations [3]. The pharmacological activities of the plant include anti-arthritic, anti-aging, nervine tonic, cognitive function improvement in geriatric state and recovery from neurodegenerative disorders. Molecular pharmacological investigations have elucidated the association of these activities of the herb with its specific secondary metabolites known as withanolides [4]. Withanolides are C_{28}-steroidal lactones based on an intact or rearranged ergostane frame through appropriate oxidations at C-22 and C-26 to form a δ-lactone ring, and are chemically called 22-hydroxy ergostane-26-oic acid 26,22-lactone. Withaferin A (WA) is one of the major and most predominant withanolides found in the plant.

Although the biological activity of crude root extracts of this plant has been frequently reported [3–8], only few of them concern with the pure compound WA [9–15]. However, its effect on the leukemic cells of lymphoid origin, especially in the primary cells of children with acute lymphoblastic leukemia (ALL) or patients with myeloid origin, has not been addressed earlier. The signaling pathways underlying WA-induced apoptosis of these leukemic cells are yet to be established.

In the present study, we first assessed the effect of WA on several malignant cell lines. Our results indicated that WA could induce potential anti-proliferative activity on the cell lines of ALL and human myelogenous leukemia. Moreover, the results also demonstrated that WA induces apoptosis preferentially in the leukemia cells and found to be associated with (i) mitochondrial instability-mediated apoptotic signaling orchestrated by a time-dependent down regulation of the Bcl-2/Bax ratio, (ii) release of cytochrome *c* from mitochondria, (iii) activation of caspases (9 and 3) and (iv) subsequently accumulation of cells in the sub-G0 phase followed by DNA fragmentation.

The molecular mechanism of WA-induced apoptosis in these cells was mediated by p38MAPK signaling pathway. The activation of p38MAPK caused an increase in phosphorylated p38MAPK expression, which further activated downstream signaling by phosphorylating the transcription factors ATF-2 and HSP27 in WA-induced cells. The RNA interference of p38MAPK caused an inhibition of (i) active phosphorylation of p38MAPK, (ii) Bax level, (iii) activation of caspase 3 and (iv) Annexin V positivity demonstrating reduced WA-induced apoptosis.

More importantly, WA was capable of inducing apoptosis in the primary cells of patients with B-, T-ALL and myeloid leukemias with no toxicity in normal human lymphocytes and primitive hematopoietic progenitor cells. Our studies demonstrate that WA-induced apoptosis triggers p38MAPK signaling pathway to activate apoptotic pathway and increase the levels of Bax-initiated mitochondrial death cascade, suggesting its potential for the treatment of both lymphoid and myeloid leukemia.

Materials and methods

Chemicals

Fetal bovine serum (FBS) was purchased from Gibco-BRL. Propidium iodide (PI) and JC1 (5,5′,6,6′-tetrachloro-1,1′,3,

3′-tetraethylbenzimidazolylcarbocyanine iodide) were purchased from Molecular probes. Fluorescein isothiocyanate (FITC)-annexin V, benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl keton (Z-VAD-fmk), monoclonal antibodies (Mab) against Bax, BCl-2, cytochrome *c*, caspase 3, procaspase 3, caspase 9, poly (ADP-ribose) polymerase (PARP), p38 MAPK and Apo Direct^{TM} kit were purchased from BD biosciences. Phospho p38 MAPK pathway sampler kit containing polyclonal antibodies against Phospho p38 MAPK, phospho HSP27 and phospho ATF-2 was from Cell Signal Technologies. P38 MAPK inhibitor SB203580 was from EMD chemicals. All the primers used in this study were from Integrated DNA Technologies (USA). All other chemicals unless otherwise stated were from Sigma Chemical Company (St. Louis, MO).

Cell lines and cell culture

Human cell lines, including acute T (MOLT-4 and Jurkat), acute B lymphoblastic leukemia (REH), chronic myeloid leukemia (K-562), epithelial adenocarcinoma (HeLa) and osteogenic sarcoma (Saos-2), and mouse myeloma cell line SP2/0 were obtained from American Type Culture Collection (Manassas, VA). The cells were grown in RPMI or DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic mixture (Medium A), and cultured at 37°C in a humidified incubator containing 5% CO_2.

Cord blood mononuclear cells were isolated by means of Ficoll density gradient centrifugation. $CD34^+$ lineage negative ($CD34^+lin^-$) enriched population was isolated using human primitive progenitor enrichment kit (Stem Cell Technology, West Broadway, Canada) according to the manufacturer's instructions.

Clinical sample

Clinical samples (1.0 ml) from confirmed patients with ALL ($n = 13$) and myeloid ($n = 5$) at Ramkrishna Seba Prathisthan (Kolkata, India) were collected before chemotherapy and sent to the Indian Institute of Chemical Biology [16]. The diagnosis was established on the basis of morphological examination and immunophenotyping using commercially available antibodies against established markers whose expressions are aberrant in leukemia. Peripheral blood mononuclear cells (PBMC) from normal healthy individuals and patients were separated by Ficoll gradient. The Institutional Human Ethical Committee had approved the study, and samples were taken with the consent of the donors, patients, or their parents or guardians.

Isolation and characterization of WA

The leaves of *Withania somnifera* (accession number NMITLI-101/National Gene Bank at CIMAP No. RS-NMITLI-II.A) were collected from the experimental farm at Central Institute of Medicinal and Aromatic Plants, Lucknow. Fresh leaves were extracted with methanol, filtered, residue was re-extracted, washed, partitioned with chloroform and chromatographed over a silica gel column [17]. The structure of purified WA (Fig. 1a) was determined by infra red, ^1H and ^{13}C nuclear magnetic resonance (NMR), two-dimensional NMR, and mass spectroscopy. The purity (>99%) of the compound was ascertained with high-resolution TLC and reverse-phase HPLC [18].

Cytotoxic effect of WA on lymphoblasts

The cytotoxicity of WA against lymphoblasts was estimated by trypan blue dye exclusion, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and [^{3}H]thymidine incorporation assays. Cells (2×10^4/250 µl/well) in the log phase were seeded on 96-well tissue culture plates and incubated with freshly prepared WA (0–30 µM) in duplicate for 24–72 h at 37°C in a humidified atmosphere containing 5% CO_2. Cell viability was assessed by counting the viable cells in each well in triplicate under a light microscope.

The cells were seeded for designated time in the presence and absence of WA. [^{3}H]thymidine (0.1 µCi) was added 16–18 h before harvest time. Radioactivity was

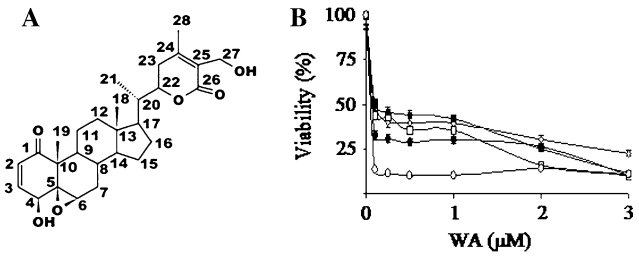


Fig. 1 WA triggers decrease in cell viability in leukemic cells. **a** Structure of WA. WA was purified from leaf of *Withania somnifera* and exhibited the following characteristics: m.p. 252–253°C, M.W 470, $[\alpha]_D^{28}$ +125° ($CHCl_3$,c 1.30). The homogeneity of the compound was determined by TLC and HPLC and the structure of WA was established by infra red, ^1H and ^{13}C nuclear magnetic resonance (NMR), two-dimensional NMR, and mass spectroscopy through direct comparison with an authentic sample. **b** WA induced reduced cell viability on leukemic cells. MOLT-4 (-◆-), Jurkat (-O-), REH (-◇-), K562 (-■-) and Saos-2 (-□-) cells (2×10^4/250 µl/well) in complete RPMI were seeded on 96-well tissue culture plates and incubated with WA (0–3 µM) in duplicate for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. [^{3}H]thymidine (0.1 µCi) was added 16–18 h before harvest time. Radioactivity was measured using a liquid scintillation counter. Each point corresponds to the mean ± SD of three experiments in duplicates

measured using a liquid scintillation counter (Packard Bioscience, USA). The viability of treated and untreated cells was also checked by MTT assay as described elsewhere [19]. Each experiment was performed at least three times and in duplicate.

Admix experiment

In order to achieve a defined blast population in vitro, normal PBMC and MOLT-4 cells were mixed proportionately to get a blast percentage between 10% and 100%, and thereafter the mixed cell (2×10^4) was similarly seeded with WA (3.0 μM) and incubated for 24 and 48 h. Cell viability was checked using trypan blue dye exclusion assay.

Colonogenic assay

WA (3.0 μM)-pretreated cells (2.5 h) were washed with Medium A, then rapidly suspended in Medium A (2 ml) containing bactoagar (0.36%) at 37°C, and spread into 35-mm dishes containing a hard agar layer (0.72%). The plates were incubated at 37°C for 2 weeks, and the colonies were counted under a microscope.

Flow cytometric analysis of externalized phosphatidylserine

The cells were incubated with WA (3.0 μM) for 0–24 h, centrifuged, washed twice in phosphate buffer saline (0.02 M, pH 7.2, PBS) and resuspended in Annexin V binding buffer according to the manufacturer's instructions [20]. FITC-Annexin V and propidium iodide were then added and processed. Data acquisition was done on a FACSCalibur flow cytometer (BD) and analyzed with CellQuest software.

Flow cytometric determination of cell cycle status

MOLT-4 cells after WA (3.0 μM) treatment for 0–24 h were fixed in ice-cold methanol (70%) for 2 h, washed in PBS and incubated with propidium iodide (50 μg/ml) in the presence of RNAse (200 U/ml). The percentage of cells in different phases of the cell cycle was determined. WA-treated normal PBMC was evaluated similarly.

Flow cytometric determination of status of Bcl-2 and Bax

The intercellular levels of Bcl-2 and Bax in WA-treated cells were measured using PE-Bcl-2 and FITC-Bax antibodies. The ratio of Bcl-2 and Bax was calculated from mean fluorescence intensity and plotted against different time points.

Measurement of mitochondrial trans-membrane potential ($\Delta\psi_m$)

The cells were incubated at 37°C in the absence or presence of WA (3.0 μM) for 0–20 h, washed in PBS, centrifuged, resuspended in PBS, probed with a cell permeable dye (JC1; 10.0 μg/ml) and incubated at 25°C for 10 min [21]. The relative ratio of emission of red (585 nm) and green (530 nm) fluorescence from mitochondria and cytosol, i.e. polarization of $\Delta\psi_m$, was analyzed immediately by flow cytometry.

Activation of caspases

The activation of caspase 3 in WA (3.0 μM)-treated MOLT-4 and REH cells for 0–24 h as well as for cells pretreated with caspase inhibitor Z-VAD-fmk (10.0 μM) was analyzed. The cells were washed, fixed, permeabilized with cytofix/cytoperm and incubated with phycoerythrin (PE)-conjugated Mab against active caspase 3 in the presence of 2% BSA and 1% saponin [22]. The percentage of PE-positive cells was determined by flow cytometry. The activation of caspase 3 and 9 was also measured by fluorimetric assay kit (BioVision) according to the manufacturer's protocol by fluorimetric method.

In situ detection of DNA fragmentation by terminal deoxynucleotidyltransferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) end labeling (TUNEL)

DNA fragmentation within the cell was analyzed using the apo-direct kit (BD) following the manufacturer's instructions. In brief, WA-treated cells were fixed in paraformaldehyde, washed, incubated in ice-cold ethanol, washed, reacted with TdT enzyme and stained with propidium iodide and FITC-dUTP and processed for flow cytometry.

p38MAPK phosphorylation

Phosphorylation of p38MAPK of untreated or WA-treated MOLT-4 cells along with cells pre-treated with a pharmacological p38MAPK inhibitor SB203580 was analyzed by flow cytometry. After incubation, the cells were fixed, permeabilized, incubated with anti-phospho p38MAPK Mab in the presence of 2% BSA and 1% saponin and stained with FITC-secondary antibody. The percentage of FITC-positive cells was determined.

The role of p38MAPK by p38MAPK siRNA

Cells maintained in Medium A were plated at 50% confluence in a 12-well plate. They were transfected with pool p38MAPK siRNA with a transfection reagent following the manufacturer's instructions. The following day, the siRNA mixture was removed and replaced with a fresh medium. The efficiency of transfection was checked by FACS analysis using FITC-siRNA. Transfected and non-transfected cells were incubated with WA (3.0 μM) for 24 h. The induction of apoptosis was checked by FITC-Annexin V binding assay. Non-transfected cells pre-treated with SB203580 were evaluated similarly.

Western blot analysis

The cells (MOLT-4, 1×10^7) were incubated without or with WA (3.0 μM) for 0–24 h, washed with cold PBS and homogenized in a glass homogenizer. Mitochondria and cytosol were separated by differential ultracentrifugation [23]. Equal amounts (30 μg) of each fraction were separated by SDS-PAGE (10%) and electrotransferred onto a nitrocellulose membrane [24, 25]. Blots were blocked with PBS containing 2% BSA, washed with PBS containing 0.1% Tween 20 and probed using appropriate antibodies by incubating overnight at 4°C. The immunoreactive protein was detected by incubating the blots for 1 h at 37°C with peroxidase-conjugated secondary antibodies and developed with diamino benzidine. Similarly, the pre-treated cells (1 h) with different inhibitors of the apoptotic pathway were exposed to WA and processed. The Western blot analysis was done for Bcl-2, Bax, cytochrome *c*, procaspase 3, caspase 3, caspase 9, PARP, p38MAPK, p-p38MAPK, p-ATF2 and p-HSP27.

Genetic expressions

In order to check the altered expression level of different genes involved in apoptosis after WA treatment (3 μM) of MOLT-4 cells at different periods, the total RNA was isolated by RNeasy Mini Kit (Qiagen) as recommended by the manufacturer. Initially, cDNAs were synthesized by Im-Pro-II-Reverse Transcription System (Promega) and used as template for PCR according to the following protocol: 30 s at 94°C, 35 s at 52°C, 35 s at 72°C for 40 cycles, followed by 4 min at 72°C for different primers. The following primers were used for real-time and semi-quantitative PCR analysis—for Bcl-2:5′-GTG ACT TCC GAT CAG GAA GG-3′ (forward), 5′-CTT CCA GAC ATT CGG AGA CC-3′ (reverse); Bax: 5′-AGT AAC ATG GAG CTG CAG AGG (forward), 5′-ATG GTT CTG ATC AGT TCC GG-3′ (reverse); caspase 3:5′-TCG GTC TGG TAC AGA TGT CG-3′ (forward), 5′-CAT ACA AGA AGT CGG CCT CC-3′ (reverse); β-actin: 5′-CGA CAG GAT GCA GAA GGA AG-3′ (forward), 5′-ACA TCT GCT GGA AGG TGG A-3′ (reverse). The PCR products were electrophoresed on a 1% agarose gel and stained by ethidium bromide under UV light. In parallel, real-time PCR was also performed by taking first-strand cDNAs of the above preparation. The expression of different genes was normalized by the expression of the housekeeping gene β-actin.

Data analysis

Data were from at least three independent experiments. Statistical analysis of data was performed using two-tail student *t*-test. Errors bars represent standard error of mean (SEM). *** represent the $P < 0.001$.

Results

WA inhibited cell growth on several malignant cell lines of lymphoid and myeloid origin

WA isolated from *Withania somnifera* was found to exhibit anti-proliferative effect on leukemic cells of lymphoid and myeloid origin in dose- and time-dependant manner. WA was evaluated using a 2-mM stock solution in 50% ethanol diluted in the culture medium using trypan blue exclusion method, radioactive thymidine incorporation and MTT assay. It exerted substantial growth inhibition (>80%) of human T-(MOLT-4, Jurkat) and B-(REH)-ALL cell lines at a concentration of 3.0 μM after 48 h (Fig. 1b). After 72 h of treatment, no viable cells were found. Under identical conditions, significant cell death was observed on leukemic cells of myeloid origin (K-562), suggesting the ability of WA to destroy myeloid cells in equal capacity. The IC_{50} values for MOLT-4, Jurkat, REH and K562 cells were within the range of 0.25–1.92 μM (Table 1). The viability of all cell lines was only about 2–10% as analyzed by [^{3}H]thymidine incorporation after 60 h of WA treatment. The percentages of viability of MOLT-4, Jurkat and REH cells were only $6 \pm 4\%$ after 72 h of WA (3.0 μM) treatment when analyzed by semi-automated MTT assay. WA also showed extensive anti-proliferative effect on three other cancer cell lines, e.g. Saos 2, HeLa and murine hybridoma cell line (SP2/0), by all three methods, indicating its potential application as board-spectrum anti-cancer agent.

Additionally, the anti-proliferative activity of WA was demonstrated by colonogenic assay. Clonogenicity of a representative MOLT-4 cell line was drastically reduced after exposure to WA (3.0 μM) as compared to untreated cells under identical conditions (figure not shown). Most of the WA-exposed cells either died or remained as single cells after 2 weeks on agar.

Table 1 IC_{50} of different leukemic cell lines and leukemic patients

Leukemic cell	IC_{50}[a] (μM)	IC_{50}[b] (μM)
REH (B-ALL)	0.40	3.09
MOLT-4 (T-ALL)	0.33	1.52
Jurkat (T-ALL)	0.25	1.62
K562 (chronic myelogenous leukemia, human)	0.43	0.58
Saos-2 (osteosarcoma, human)	0.38	1.75
HeLa (epitheloid carcinoma, human)	1.92	1.65
SP2/0 (myeloma, mouse)	1.52	1.5
B-ALL patients (Blast 75 ± 6%, $n = 6$)	0.54 ± 0.03	1.35 ± 0.5
T-ALL patients (Blast 80 ± 4%, $n = 4$)	0.58 ± 0.028	2.4 ± 0.6
Myeloid leukemic patient (Blast 70 ± 10%, $n = 5$)	0.68 ± 0.033	1.85 ± 0.7

[a] Determined by the treatment of leukemic cells with WA for 48 h. [^{3}H]thymidine was added 16–18 h before harvest time point and processed as described in section "Material and method"

[b] Determined by trypan blue dye exclusion

Induction of apoptosis by WA in human leukemia cells

WA-induced apoptosis caused externalization of phosphatidylserine

The appearance of phosphatidylserine on the external surface of plasma membrane serves as a marker for programmed cell death (apoptosis). Accordingly, we investigated whether WA-induced killing of leukemic cells was due to apoptosis. The mode of cell death in WA (3.0 μM)-treated MOLT-4 and REH cells was monitored for 0–24 h by staining cells with FITC-Annexin V. The loss of plasma membrane integrity was followed by the uptake of propidium iodide. A time-dependant increase in the binding of Annexin V was observed (Fig. 2a). The staining of MOLT-4 and REH cells with Annexin V and its absence in untreated cells support a specific apoptotic effect of WA. Annexin V binding was detectable as early

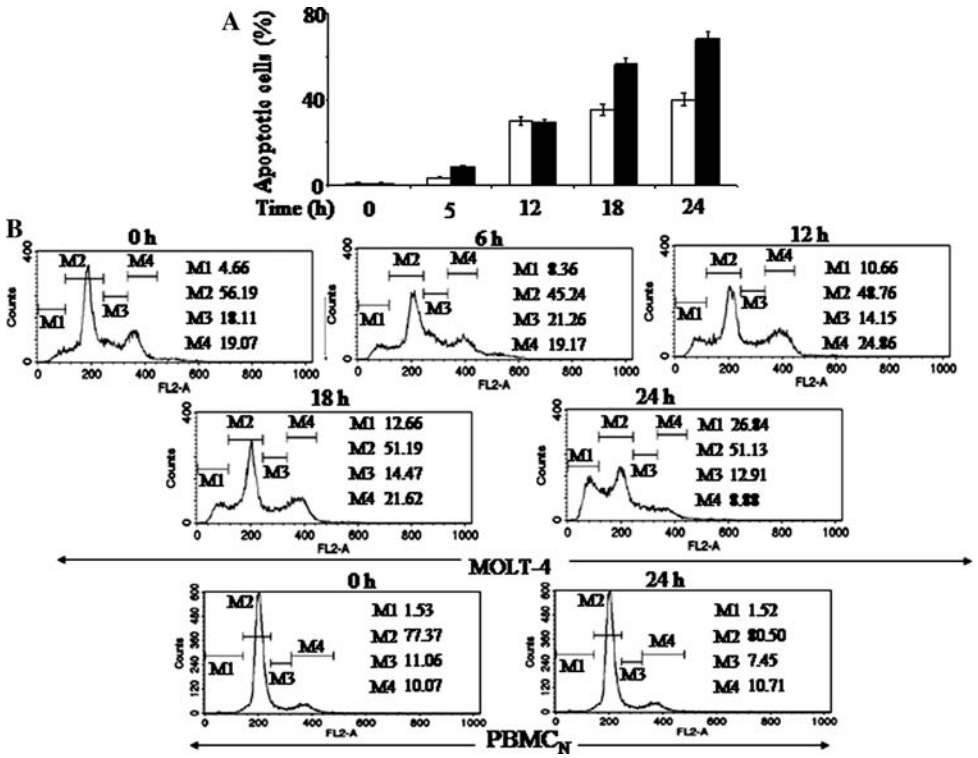


Fig. 2 WA induced externalization of phosphatidylserine by flow cytometry. **a** MOLT-4 (□) and REH (■) cells were incubated with WA (3.0 μM) for 0-24 h and stained with FITC-Annexin V and propidium iodide (PI). Apoptotic cells (%) versus time (h) of exposure (WA) have been shown. **b** WA induced apoptosis in leukemic cells by cell cycle analysis. MOLT-4 cells were incubated with WA (3.0 μM) for 0–24 h for DNA cell cycle analysis. The cells were analyzed through FACS analysis after propidium iodide staining. Gates were set to assess % of cells in sub G0-G1 (<2n DNA, M1), G1 (2n DNA, M2), S (>2n DNA, M3) and G2+M (4n DNA, M4). *Bars* denote the boundaries of cell cycle phases. This is a representative profile of at least three experiments. WA-treated normal PBMC ($PBMC_N$) was evaluated similarly

as 12 h after treatment. After 24 h of WA exposure, 39.9% and 67.9% of MOLT-4 and REH cells, respectively, were positive. A clear ring of green fluorescence around MOLT-4 cells was evident after 24 h of treatment, which was absent in untreated cells as seen by confocal microscopy (figure not shown).

WA-induced apoptosis was associated with enhanced accumulation of cells in the sub-G0 phase

To confirm whether growth inhibition observed in WA-treated leukemic cells was caused by the induction of apoptosis, the DNA cell cycle of MOLT-4 cells was examined in the presence WA (3.0 μM) for 0–24 h. The cells were analyzed through FACS analysis after propidium iodide staining [26]. In WA-treated cells, a time-dependent dramatic increase in the accumulation of apoptotic or dead cells in the sub-G0 region was observed to continue up to 24 h (26.84%, Fig. 2b) based on DNA degradation. A similar observation was found in leukemic patients (data not shown). However, there was no effect on leukemic cells in the absence of WA. The proportion of apoptotic cells was almost unaltered in normal PBMC when treated with WA under identical conditions. Cells treated with ethanol for 24 h were used as control.

WA induced enhanced expression of *Bax*

Among the mitochondrial factors, anti-apoptotic factor Bcl-2 and pro-apoptotic factor Bax are two essential elements involved in cell survival and cell death. Proapoptotic Bax undergoes a conformational change and accumulates on the mitochondrial surface triggering the release of cytochrome *c* to activate the final step of apoptosis. Therefore, to further understand the role of WA-induced apoptosis in leukemic cells, we studied whether WA has any effect on the expression of these two proteins. Accordingly, the expression of these pro-apoptotic and anti-apoptotic members of the family was studied by Western blot (Fig. 3a), RT-PCR (Fig. 3b), real-time PCR analysis (Fig. 3c–e) and FACS (Fig. 3f) in WA-treated cells for 0–24 h. Increased band intensity of Bax were observed in the mitochondrial fraction of WA-treated MOLT-4 cells in time-dependant manner (Fig. 3a). In a parallel set of experiments, staining with anti-Bcl-2 antibody revealed a strong band in untreated leukemic cells. A time-dependent decrease in Bcl-2 band intensity in the cell lysates of WA-treated MOLT-4 cells along with a sharp decrease in the ratio of Bcl-2 to Bax with time was observed in FACS analysis (Fig. 3f). The maximum decrease was after 24 h of WA exposure. This observation was further corroborated by semi-quantitative RT-PCR (Fig. 3b). A time-dependant decrease and increase of gene expression in Bcl-2 and Bax, respectively, were observed. Quantitative measurement of the gene expression of Bcl-2 (Fig. 3c) and Bax (Fig. 3d) by real-time PCR also showed a time-dependant decrease of Bcl-2/Bax ratio (Fig. 3e) as corroborated by FACS analysis (Fig. 3f). Therefore, a decrease in the survival rate of these WA-induced leukemia cells was noted to be associated with a decrease in the Bcl-2/Bax ratio suggesting damages in mitochondria by affecting these Bcl-2 family proteins.

WA-induced apoptosis is mitochondria-dependent

Decrease in mitochondrial trans-membrane potential ($\Delta\psi_m$)

To investigate the alteration of $\Delta\psi_m$ in WA (3.0 μM)-treated cells, a time course study was performed. Mitochondrial involvement in WA-treated MOLT-4 cells was characterized by FACS analysis (Fig. 3g) using JC1, which undergoes a transition from molecular aggregation to molecular monomer formation as detected by a shift in fluorescence from red to green during depolarization of $\Delta\psi_m$. On exposure of the cells to WA, the ratio of 585/530 nm fluorescence ($\Delta\psi_m$), i.e. J-aggregates, in mitochondria versus monomers in the cytosol showed a decrease after 8 h of treatment, and this continued up to 20 h suggesting depolarisation of $\Delta\psi_m$.

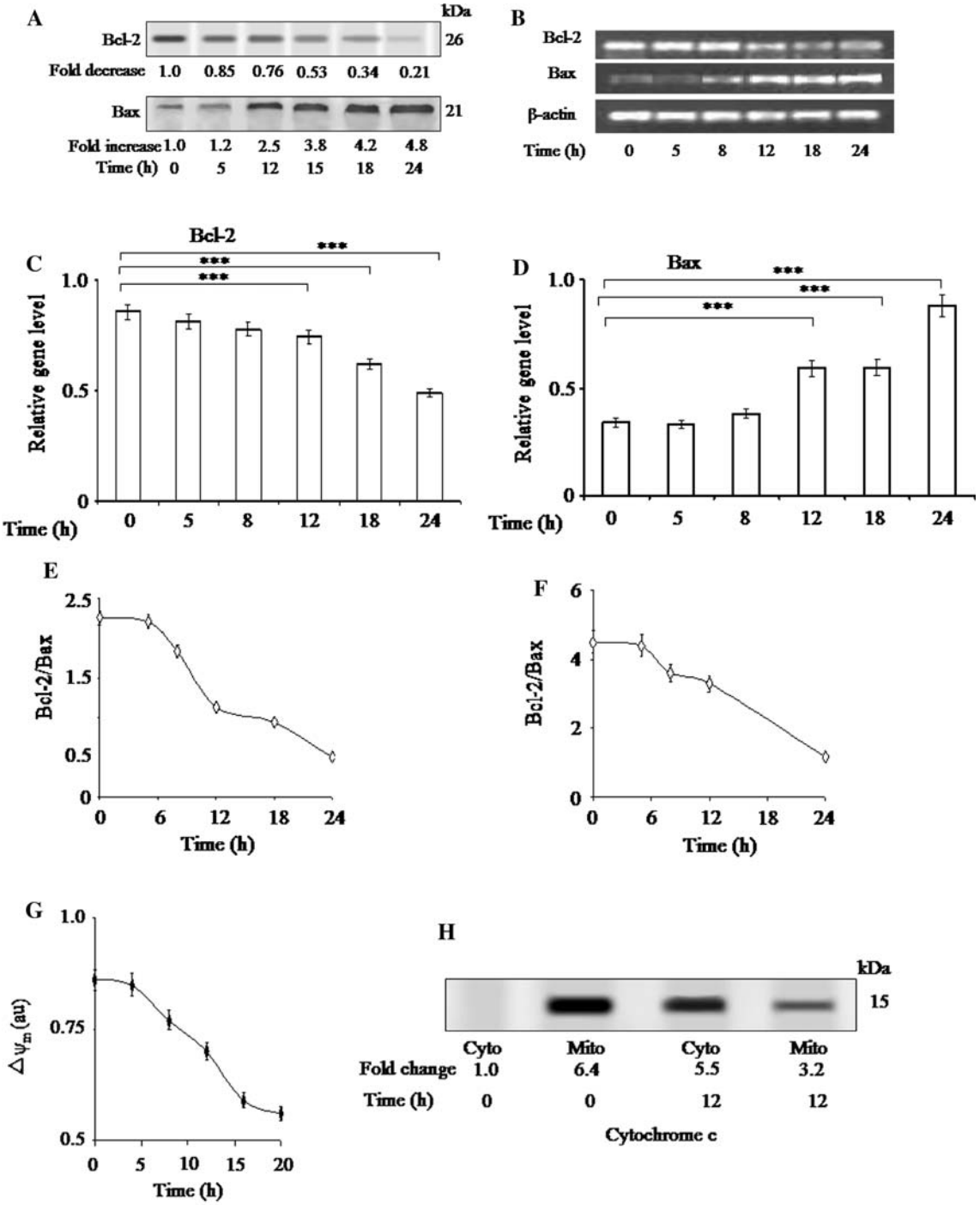
MOLT-4 cells always appeared with plenty of red spots (J-aggregates) when stained with JC1 and placed under a confocal microscope (figure not shown). At the same time, WA-treated cells (12 h) showed up faint green balls with very few reddish spots indicating reduction in $\Delta\psi_m$.

WA induced the release of cytochrome c

During apoptosis, a decrease in $\Delta\psi_m$ prompts cytochrome *c* to be released in the cytosol from mitochondrial trans-membrane space. To confirm whether reduction in $\Delta\psi_m$ observed in WA-treated leukemic cells was able to release cytochrome *c*, MOLT-4 cells were exposed to WA (3.0 μM) for 0–18 h. The release of cytochrome *c* from mitochondria to cytosol was detected by Western blot analysis using anti-cytochrome *c* antibodies. The maximum release of cytochrome *c* in cytosol was observed after 12 h of treatment with WA, which possibly triggered cell apoptosis (Fig. 3h). However, cytochrome *c* in cytosol was absent in untreated cells. Approximately two-fold less cytochrome *c* was detected in mitochondria of WA-treated cells compared to untreated cells.

Activation of caspases in WA-treated leukemic cells

The release of cytochrome *c* from mitochondria can lead to the activation of caspase 9 and then caspase 3.



Accordingly, caspase-dependent apoptotic cell death features caspase activation. In order to determine whether caspase 9 and caspase 3 are really cleaved during WA-induced apoptosis in MOLT-4 cells on exposure to WA (3.0 μM) for 0–24 h, Western blot analysis was performed (Fig. 4a). The antibody we employed for caspase 9 recognized both pro and active forms; however, anti-caspase 3 Mab was able to detect only the active form. As shown in the time course in Fig. 4a, WA exposure resulted in the processing and cleavage of both caspase 9 and caspase 3. The active form of caspase 9 was maximum after 18 h of WA treatment. The processing of the pro-form of caspase 3 (p32) to the active form of caspase 3 (p18) was also observed in the cell lysate of MOLT4.

The activation of caspases was further corroborated by FACS analysis (S1), fluorimetric measurement (Fig. 4b–c),

◀**Fig. 3** WA induced Bax-dependent mitochondrial death cascade MOLT-4 cells were treated with WA (3.0 μM) for 0–24 h and following experiments were carried. **a** A time-dependent increase in Bax band intensity in the mitochondrial fraction and decrease in Bcl-2 band intensity in the cell lysates of WA-treated MOLT-4 cells in time-dependant manner were determined by Western blot analysis using anti-Bax (1:100) and anti-Bcl-2 antibodies (1:100) as described in materials and methods. **b** Upregulation of Bax and down regulation of Bcl-2 mRNA expression. The Bcl-2 and Bax gene level was evaluated by semi-quantitative RT-PCR by using cDNA (5 μg) from WA-treated MOLT-4 cells at different time periods. The genetic expression was normalized by the expression of the housekeeping gene β-actin. Real-time PCR analysis of Bcl-2 (**c**) and Bax (**d**) mRNA level were determined. The data are the means ± SD of five different experiments. Significance according to two tail student t test $P < 0.001$ is shown by '***'. **e** Real-time PCR showed a time-dependant decrease of Bcl-2/Bax ratio with time. **f** The intercellular levels of Bcl-2 and Bax in WA-treated cells were measured using PE-Bcl-2 and FITC-Bax antibodies by FACS analysis. The ratio of Bcl-2 and Bax was calculated from mean fluorescence intensity and plotted against different time point. The data represent means ± SD of three experiments. **g** WA-induced apoptosis in leukemic cells was mitochondria dependent. Mitochondrial involvement in WA-treated MOLT-4 cells was characterized using a dye JC 1 as described in section "Material and methods". The relative ratio of 585/530 nm fluorescence ($\Delta\psi_m$) i.e. J-aggregates in mitochondria versus monomers in the cytosol represents $\Delta\psi_m$. The ratio of emission of red (585 nm) and green (530 nm) fluorescence from mitochondria and cytosol, i.e. polarization of $\Delta\psi_m$, was analyzed immediately by flow cytometry and shown as a plot of $\Delta\psi_m$ with time. **h** Release of apoptotic cytochrome *c*. The release of cytochrome *c* from mitochondria (Mito) to cytosol (Cyto) was detected by Western blot analysis using anti-cytochrome *c* antibodies before and after 12 h of treatment with WA. Untreated cells served as control

RT-PCR (not shown in figure) and quantitative PCR (S2). On exposure of leukemic cells to WA, the binding of PE-conjugated Mab to active caspase 3 showed a time-dependant increase in caspase 3 activation being 32.40% after 24 h of WA treatment (S1). When the cells were pretreated with a broad-spectrum caspase inhibitor like Z-VAD-fmk, caspase antibodies showed reduced binding in WA-treated cells, substantiating the specificity of noted caspase activation.

This observation was supported by the fluorimetric measurement of relative caspase 9 and caspase 3 activities. Although caspase 9 activity in WA-treated MOLT-4 cells increased (Fig. 4b), a dramatic stimulation of executioner caspase 3 activity was observed to peak at 24 h (Fig. 4c). This stimulation of caspase 3 activity was much more pronounced than that of caspase 9. It has been demonstrated by a drastic 20.45-fold increase in relative caspase 3 activity as compared to only 4.0-fold increase of caspase 9. A similar type of caspase activation was observed in REH cells after WA treatment.

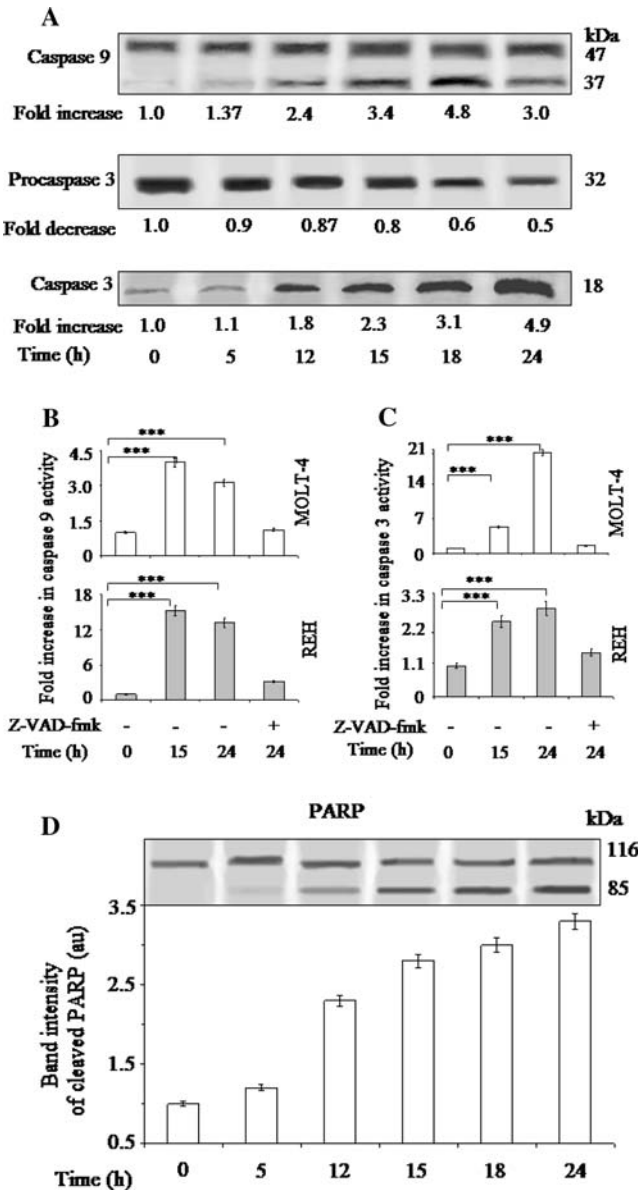


Fig. 4 WA-triggered cell death involves cleavage of caspases. ▶ **a** Activation of caspase-dependant death. In order to determine whether caspase 9 and caspase 3 are really cleaved during WA-induced apoptosis in MOLT-4 cells on exposure to WA, Western blot analysis was performed as described in section "Materials and methods". The antibody we employed for caspase 9 recognized both pro and active forms; however, anti-caspase 3 Mab was able to detect only the active form. WA-exposed cells showed enhanced activation of caspase 9 and 3. The activation of caspase 9 (**b**) and caspase 3 (**c**) in WA-treated MOLT-4 and REH cells for 0–24 h as well as for cells pre-treated with caspase inhibitor Z-VAD-fmk (10.0 μM) was analyzed by fluorimetric method (BioVision kit) using fluorescently labeled substrates according to the manufacturer's protocol. They are expressed in terms of fold increase compared to untreated cells. The data shown are from a representative experiment performed three times with comparable results. The values reflect the mean ± SD of triplicate determination. **d** Cleavage of PARP in WA-induced leukemic cells. The cleavage of PARP in WA-treated (3.0 μM) MOLT-4 cells for 0–24 h was determined by Western blot analysis using anti-PARP antibodies (1:100, over-night) as described in section "Materials and methods". Anti-PARP Mabs were capable of detecting both pro and active forms. The intensity of band (arbitrary unit) has been plotted against time (h)

This finding was fully supported by a significant time-dependant increase in relative gene expression of caspase 3 in WA-treated cells. The treatment of MOLT-4 cells with WA for 0–24 h demonstrated a progressive time-dependant increase in mRNA expression executioner caspase 3, which peaked at 24 h as demonstrated by real-time PCR (S2). In contrast, minimal changes were observed in cells not exposed to WA under identical conditions.

WA induced cleavage of PARP in leukemic cells

Poly ADP-ribose polymerase (PARP, 116 kDa) is mainly involved in DNA repair. The ability of PARP to repair DNA damage is affected substantially by its cleavage through the activation of caspase 3. PARP is one of the main cleavage targets of caspase 3 in vivo. WA-induced cell death resulted in the cleavage of PARP to yield an 85-kDa cleaved fragment in the lysate of WA-treated (3.0 μM) MOLT-4 cells by Western blot analysis using anti-PARP antibodies in a time-dependent manner (Fig. 4d). Taken together, these results clearly imply that WA induces mitochondria-dependent apoptosis in MOLT-4.

WA activated the p38MAPK pathway

We have demonstrated that WA is able to induce apoptosis of leukemic cells. We examined the possible involvement of MAP kinase. p38MAPK was analyzed because of its importance in the mediation of a stress-signaling cascade [27]. The activation of p38MAPK was determined by flow cytometry using phospho-specific antibodies against p38MAPK. WA (3.0 μM) caused ~90.96% increase in phosphorylation of p38MAPK expression after 2.5 h. To investigate the role of p38 pathways on WA-induced apoptosis of leukemic cells, we examined the effect of specific p38 inhibitor SB203580 on p38 phosphorylation and apoptosis. WA-induced enhanced phosphorylation of p38MAPK in MOLT-4 cells was dramatically reduced from 90.96% to 14.23% when cells were pretreated for 1.0 h with SB203580 (10.0 μM) followed by WA treatment for 2.5 h (Fig. 5a).

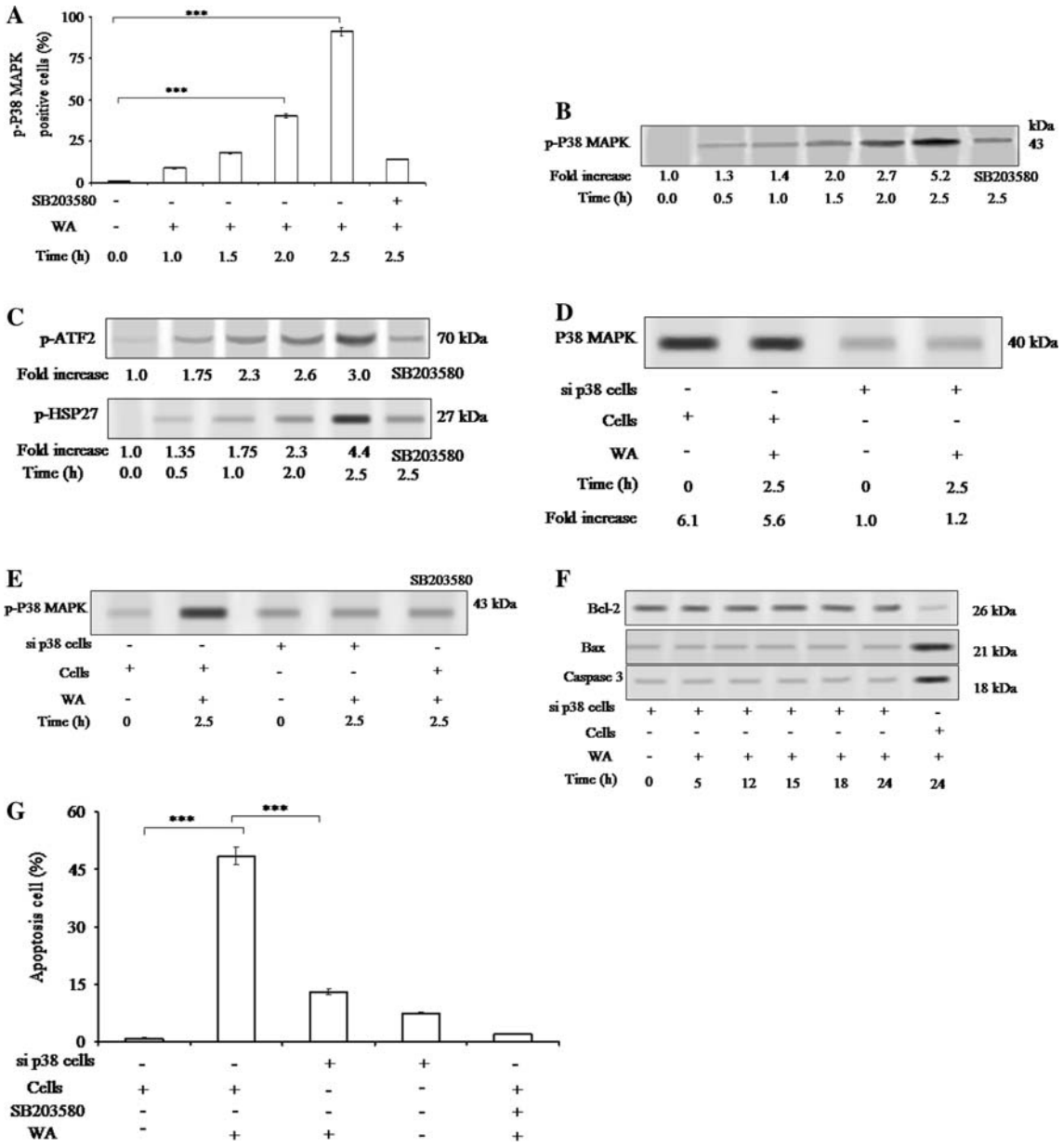
The activation of p38MAPK signaling cascade was corroborated by Western blot analysis using phospho-specific p38MAPK antibodies (Fig. 5b). Increased phosphorylation of p38 in MOLT-4 cells started after 30 minutes of WA addition, which maximized after 2.5 h, the increase being 5.2-fold as compared to untreated cells. Enhanced phosphorylation of p38 lasted approximately up to 5 h and then slowly declined with time (not shown in figure). Cells pretreated with SB203580 (10.0 μM) for 1.0 h followed by WA treatment for 2.5 h showed 63.5% reduction in phosphorylation of p38MAPK.

Fig. 5 WA-induced apoptosis by activation of p38MAPK signaling pathway Phosphorylation of p38MAPK of untreated or WA-treated MOLT-4 cells along with cells pre-treated with a p38MAPK inhibitor SB203580 was analyzed at indicated time (h). **a** FACS analysis. After incubation, the cells were fixed, permeabilized, incubated with anti-phospho p38MAPK Mab in the presence of 2% BSA and 1% saponin and stained with FITC-secondary antibody. The percentage of FITC-positive cells is shown as p-p38MAPK positive cells (%). This is a representative profile of at least 3 experiments in duplicate. Significance according to two tail student *t* test $P < 0.001$ is shown by '***'. Western blot analysis. Cytosolic fractions were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated separately with polyclonal anti-phospho p38MAPK (**b**), anti-phospho ATF-2 and (**c**) anti-phospho HSP27 antibodies (1:1000, over-night), and processed as described in the materials and methods. Confirmation of p38MAPK dependency of WA-induced apoptosis by silencing p38MAPK gene. The MOLT-4 cells were transiently transfected with p38MAPK-specific siRNA or a control non-silencing siRNA, as described in section "Materials and methods". Both transfected and non-transfected cells were incubated with WA (3.0 μM) as indicated time points. **d** The status of p38MAPK due to the transient expression of p38MAPKsiRNA was measured by Western blot analysis using Mab against p38MAPK. For comparison, the level of p38MAPK has been shown in non-transfected cells before and after treatment of WA. **e** The status of phospho p38MAPK in transfected cells before and after exposure with WA using polyclonal antibody against phospho p38MAPK by Western blot analysis. The status of phospho p38MAPK in presence of SB203580 is shown for comparison. The transfection of p38MAPKsiRNA greatly inhibited the active phosphorylation of p38MAPK (43 kDa). **f** The status of Bcl-2, Bax and active caspase 3 in p38MAPKsiRNA transfected and non-transfected cells in a time-dependant exposure of WA (3.0 μM) was determined by Western blot analysis using specific respective Mabs. The last lane represents the status of Bcl-2, Bax and active caspase 3 in non-transfected cells treated with WA as indicated time points. **g** The % of WA-induced apoptosis in p38MAPKsiRNA transfected and non-transfected cells in presence and absence of WA was determined using FITC-Annexin V positivity by FACS analysis. Cells pre-treated with the SB203580 followed by exposure of WA were shown for comparison

Activated p38MAPK in WA-treated MOLT-4 cells further activated the downstream signaling by phosphorylating the activating transcription factor-2 (ATF-2) at Thr71 and HSP27 at Ser82 (Fig. 5c). The maximum activation of ATF2 and HSP27 was observed after 2.5 h of WA (3.0 μM) treatment. Approximately 1.87- and 2.3-fold decrease in phosphorylation of ATF-2 and HSP27 was observed in cells pretreated with SB203580.

Genomic approach to confirm the involvement of p38MAPK by silencing the gene

To examine the effect of WA by blocking p38MAPK expression, we utilized the RNA interference (RNAi) technology to knock down the expression of p38MAPK. Accordingly, the MOLT-4 cells were transiently transfected with p38MAPK-specific siRNA (p38MAPKsiRNA) or a control non-silencing siRNA, and the role of p38MAPK



pathway in WA-induced apoptosis was confirmed. The transient expression of p38MAPKsiRNA showed approximately 61% reduction in p38MAPK (40 kDa) expression as compared to non-transfected cells after 24 h as measured by densitometric scanning of the Western blot (Fig. 5d). In addition, the transfection of p38MAPKsiRNA greatly inhibited the active phosphorylation of p38MAPK (43 kDa, Fig. 5e). The active phosphorylation of p38MAPK was able to inhibit the levels of Bax expression from cytosol to mitochondria and the activation of caspase 3 (Fig. 5f) in a time-dependant exposure of cells to WA (3.0 μM). Finally, siRNA knockdown of p38MAPK protected the MOLT-4 cells against WA-induced apoptosis as demonstrated by the 3.72-fold inhibition in Annexin V binding 24 h later (Fig. 5g). This observation was further corroborated by a complete inhibition of Annexin V positive cells using SB203580-pretreated cells demonstrating the specificity of the activation of p38MAPK. Taken together, these observations demonstrate a critical role for p38MAPK in WA-induced apoptosis.

WA induced apoptosis in the lymphoblasts of patients with lymphoid and myeloid origin with no toxicity on normal lymphocytes

In order to test whether the clinical samples isolated from patients were also equally susceptible to WA-induced apoptosis as observed with cell lines, lymphoblasts

(2×10^4) from children suffering from B- ($n = 6$) and T- ($n = 4$) ALL were treated with different concentrations of WA (0–30 μM) for different time periods (24, 48 and 72 h). The exposure of cells to WA led to an inhibition in cell viability in time- and dose-dependent manner (Fig. 6a). The anti-proliferative effect of WA on these lymphoblasts was almost comparable with that on ALL cell lines treated for 48 h as determined by trypan blue exclusion and [^{3}H]thymidine incorporation assays, and IC_{50} values are shown in Table 1. In parallel, the sensitivity of clinical leukemic cells from myeloid leukemia patients ($n = 5$) to WA was checked and showed comparable (IC_{50}) with that on lymphoid cells from children (Fig. 6a, Table 1).

Under similar conditions, WA did not cause any cell death on normal PBMC as monitored either by trypan blue dye exclusion or MTT assay, or even by [^{3}H]thymidine incorporation even at concentration up to 30 μM. Even the highest concentration of ethanol (0.2%) used to dissolve WA did not show any cell death in this assay. This suggested that WA exerted no major cytotoxic effect on normal lymphocytes suggesting WA specificity.

To further confirm the cytotoxicity of WA in hematopoietic progenitor cells ($CD34^+lin^-$), $CD34^+$ cells were isolated from human cord blood ($n = 3$) and lineage negative cell population was enriched. $CD34^+lin^-$ cells were exposed to WA, which exerted no major cytotoxic effect on normal hematopoietic precursors even up to 30 μM for 48 h as monitored by [^{3}H]thymidine incorporation assays (Fig. 6b). A similar result was observed when cell viability was monitored by trypan blue exclusion assay (not shown in figure).

A representative profile of the simulation of in vivo conditions of patients is shown in Fig. 6c. In a heterogeneous cell population with 0–100% lymphoblasts, WA showed the inhibition of cell viability depending on the percentage of lymphoblasts present in the mixed population. Therefore, WA is effective even when minimum number of lymphoblasts is present in normal PBMC population, suggesting its ability to distinguish cancer cells from normal cells.

Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. In this time course study, the proportion of in vivo DNA nicks was quantified by measuring the binding of FITC labeled dUTP that get attached to the nicked ends via TdT. Flow cytometry showed significant increase in the number of TUNEL-positives (Fig. 6d) in WA (3.0 μM)-exposed primary cells from patients with ALL, indicating increased apoptosis as the mode of cell death. A time-dependant increase in nuclear DNA fragmentation was observed with no significant changes in WA-treated normal PBMC.

The cleavage patterns of genomic DNA were typical of internucleosomal DNA digestion by endonucleases during apoptosis that is preceded by chromatin condensation and nuclear degradation. This was supported by the appearance of fragmentation of chromatin in more than one spot as evidenced by stained fluorescent images of WA-treated nucleus of patient's cells as observed through a confocal microscope proving nuclear fragmentation (figure not shown). In contrast, the nuclei appeared as discrete red spot in untreated cells (control) stained with propidium iodide.

Western blot of the main signaling pathway component was elucidated (Fig. 6e), which showed similar results as observed with cell lines. Based on these experimental evidences using primary cells of clinical samples from patients, it may be envisaged that WA has potential to kill lymphoblasts without affecting the normal lymphocytes as well as hematopoietic progenitor cells ($CD34^+lin^-$).

Discussion

The objectives of the present study were to demonstrate how WA induces apoptosis in leukemic cell lines and primary cells of leukemic patients, and to decipher the mechanisms and identify the key signaling molecules responsible for apoptotic cancer cell death. Although WA has been reported to exert growth inhibitory effect in several cell lines [8, 10–15], to the best our knowledge, our study has demonstrated for the first time that the natural compound WA, at very low dose and short delivery time, is equally active in exterminating leukemic cell lines and primary cells from confirmed diagnosed patients of both lymphoid and myeloid origin without affecting the normal cells. More importantly, the inertness of WA against primitive hematopoietic progenitor ($CD34^+lin^-$) cell population makes this compound even more attractive. Therefore, it is expected that WA gives comparable results in patients in vivo, suggesting its potential in clinical applications.

The pure compound, WA, established itself as anti-proliferative [8, 10], anti-angiogenesis in breast cancer cell lines [11], apoptosis-inducing activity in prostate [12] and HL-60 cells [13], and oxidative stress inducing in cell lines [13]. It induced cell death by altering the cytoskeletal structure in fibroblast and HepG2 cells [14]. WA inhibited TNF-induced NF$\kappa\beta$ activation in murine fibrosarcoma and human embryonic kidney cell lines [15]. Except for these observations, no report on p38MAPK signaling pathways activated by WA is available in literature. Accordingly, we explored the role of p38MAPK signaling pathway in WA-induced apoptosis of leukemic cell lines and primary cells of patients with ALL. We demonstrated that WA-induced cell death is dependent on p38MAPK signaling pathway through mitochondrial death cascade, and plays a critical role in inducing apoptosis. This conclusion has been

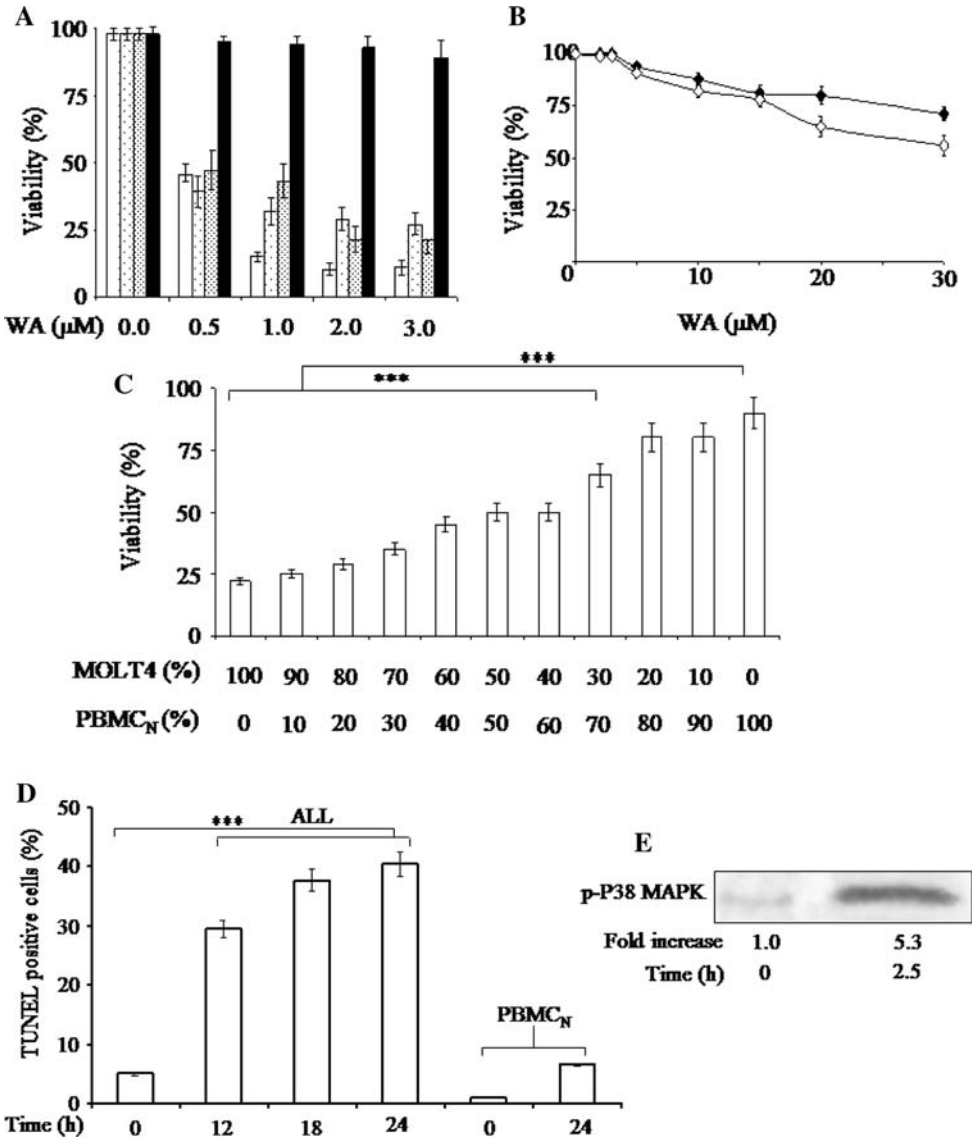


Fig. 6 WA induced apoptosis on lymphoblasts of lymphoid and myeloid origin from clinically confirmed patients. **a** Lymphoblasts from patients suffering from B-ALL (-□-, $n = 6$), T- (-▫-, $n = 4$), myeloid leukemia (-■-, $n = 5$) and normal healthy individual (-■-, $n = 5$) in complete RPMI were seeded on 96-well tissue culture plates (2×10^4/250 μl/well) and incubated with WA (0–3 μM) in duplicate for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. [^{3}H]thymidine (0.1 μCi) was added 16–18 h before harvest time. Radioactivity was measured using a liquid scintillation counter. Each point corresponds to the mean ± SD of three experiments in duplicates. **b** Cytotoxic effect of WA on hematopoietic progenitor cells. CD34$^+$ cells were isolated from human cord blood ($n = 3$) and lineage negative cell population (lin$^-$) was enriched. Hematopoietic progenitor (CD34$^+$lin$^-$) cells were exposed to WA (0–30 μM) for 24 h (◆) and 48 h (◇) at 37°C in a carbon dioxide incubator and the viability was checked by [^{3}H]thymidine incorporation assay as describe in material and methods. **c**. Apoptotic sensitivity of WA towards ALL-lymphoblast. In order to achieve a defined blast population in vitro, normal PBMC (PBMC$_N$) and MOLT-4 cells were mixed proportionately to get a blast percentage between 10 and 100%, and thereafter the mixed cell (2×10^4) was similarly seeded with WA (3.0 μM) and incubated for 48 h. Cell viability was checked using trypan blue dye exclusion assay. The data are the means ± SD of five different experiments. Significance according to two tail student t test $P < 0.001$ is shown by '***'. **d** WA-exposed primary cells from patients showed DNA nicking. DNA fragmentation within WA-treated cell was analyzed using propidium iodide and FITC-dUTP and processed for flow cytometry following the manufacturer's instructions of the apo-direct kit (BD). FITC-dUTP$^+$ i.e. TUNEL positive cells were regarded as apoptotic cells and shown in bar graph. WA-treated normal PBMC (PBMC$_N$) served as control. This is a representative profile of at least three experiments. Significance according to two tail student t test $P < 0.001$ is shown by '***'. **e** Activation of p38MAPK signal in patient cells. Primary cells from a representative B-ALL patient were incubated with WA (3.0 μM) in duplicate for 0–2.5 h. The status of phospho p38MAPK was determined by Western blot analysis using specific polyclonal anti-phospho p38MAPK (1:1000) antibodies

supported by many observations. WA-induced enhanced phosphorylation of p38MAPK in leukemic cells was followed by the activation of phosphorylated ATF-2 and HSP27. The inhibition of p38MAPK activation both by specific inhibitor and silencing of p38MAPK resulted in a dramatic protection of these cells from WA-induced apoptosis. Such a decrease in apoptosis after silencing of p38MAPK was accompanied by reduced level of Bax expression and decreased activation of caspase 3 along with unchanged Bcl-2.

The p38MAPK activity has been reported to be associated with apoptotic induction in several cell types and in response to a multitude of cellular stress [28]. ATF-2 is one of the targets for p38MAPK. Therefore, signaling pathways that regulate its activation have become a major focus of drug development programs. In the p38MAPK signaling cascade, signaling is initiated by phosphorylation of p38MAPK at Thr180, Tyr182 and subsequently HSP27. Such phosphorylation may be responsible for a change in the tertiary structure of HSP27 and increased concentrations that possibly modulate cell viability by actin polymerization and reorganization [29, 30]. The active p-p38MAPK also phosphorylated another important transcription factor ATF-2 in WA-treated cells, which has been implicated in the regulation of wide sets of genes that force the leukemic cells to undergo apoptosis. Taken together, our results indicate that early initiation of the p38MAPK signaling cascade within 2.5 h is a central event for the induction of apoptosis by WA.

An inhibitor which specifically blocked WA-induced enhanced phosphorylation of p38MAPK reconfirmed the phosphorylation of active p38MAPK as observed by a six-fold decrease in binding and 2.7-fold reduction in band intensity. The subsequent down regulation of phosphorylation of ATF-2 and HSP27 in cells prior to treatment with inhibitor further demonstrated the importance of p38MAPK signaling. Silencing the genetic expression of p38MAPK in cells before exposure to WA demonstrated six-fold inhibition of phosphorylation of activated p38MAPK, decreased level of Bax and no activation of caspase 3. All these events finally caused a 3.71-fold decrease in Annexin-V positivity, demonstrating the reduction of apoptosis of leukemic cells and thus explaining the key role of p38MAPK.

In the complex signaling events of apoptosis, the p38MAPK activation reduces the mitochondrial function initiated by alterations in the ratio of pro-(Bax) and anti-apoptotic (Bcl-2) members of the mitochondria causing the loss of $\Delta\psi_m$, release of cytochrome *c* and activation of caspases leading to apoptosis [31]. Bcl-2 directly or indirectly prevents the release of cytochrome *c* from mitochondria, and its BH4 domain can bind to the C terminal part of Apaf-1, thus inhibiting the association of caspase-9 with Apaf-1 and ultimately inhibiting apoptosis [32]. Our results suggest that the WA-induced activation of p38MAPK pathway not only decreases the Bcl-2 expression but also increases the level of Bax. Bax translocation to the mitochondria reduces $\Delta\psi_m$, enhances cytochrome *c* release and activates caspases [33, 34]. This effect is mediated through the formation of an apoptosome, a multi-protein complex consisting of cytochrome *c*, Apaf-1, pro-caspase 9 and ATP [35].

WA-induced p38MAPK activation reduced Bcl-2/Bax ratio, enhanced the cytosolic cytochrome *c* to form an active apoptosome, which in turn could activate executioner caspase 9 and caspase 3. This caspase 3 in the downstream cleaved the enzyme PARP, a substrate for caspases, to prevent the repair of damaged nuclear DNA. The entire process will result in increased accumulation of leukemic cells in the sub-G0 phase and DNA degradation. As primary cells from patients showed similar cell signaling mechanism in vitro, it may be envisaged that similar mechanism of WA-stimulated p38MAPK-mediated apoptosis may be functional even in vivo conditions in patients.

Despite making much progress in leukemia treatment, chemotherapeutic agents are expensive in both developing and underdeveloped countries. The most commonly used drugs are administered in a combination therapy, are often toxic to normal cells and have severe side effects, including resistance development, disease relapse and often requiring prolonged treatment. In this context, WA holds promise as a new, potential, alternative, inexpensive chemotherapeutic agent for the treatment of patients with leukemia of both lymphoid and myeloid origin by way of apoptosis via p38MAPK signaling pathway through mitochondrial death cascade.

Acknowledgments Chandan Mandal and Avijit Dutta are Senior Research Fellowship from the University Grant Commission and Indian Council of Medical Research respectively. This work received financial assistance from the Council of Scientific and Industrial Research, I.I.C.B. and Department of Biotechnology, Govt. of India. Suchandra Chowdhury and Susmita Mondal are acknowledged for their help in processing patient samples. The information provided in this report is protected by pending Indian patent application.

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